

Quantifying sources of variation in the frequency of fungi associated with spruce beetles: Implications for hypothesis testing and sampling methodology in bark beetle–symbiont relationships

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Abstract

The spruce beetle, *Dendroctonus rufipennis* (Kirby), causes landscape level mortality to mature spruce (*Picea* spp.) throughout western and northern North America. As with other bark beetles, this beetle is associated with a variety of fungi, whose ecological functions are largely unknown. It has been proposed that the relative frequencies of specific fungi associated with bark beetles may vary with ecological factors such as host species, climate, or beetle population phase. We collected ≈1000 adult spruce beetles in south-central Alaska from 1999 to 2001. We employed a variety of insect collection and microbial isolation techniques during year 1 to devise optimal conditions. In the latter 2 years, we sampled live adults excavated from overwintering galleries, and isolated fungi by dragging beetles across malt agar amended with gentamicin. We obtained 10 fungal species. We used a multilevel generalized linear mixed model to (a) develop estimates of the prevalence of each isolated fungal species in the beetle population; (b) explore factors that might explain the frequencies of association of specific fungi with adult spruce beetles, such as insect population phase or positive or negative associations with other fungi, and (c) partition the relative sources of variation in beetle–fungal associations between the hierarchical, random effect variables (i.e., nested individual insect, collecting vessel, tree, and site variables). We implemented this model using three procedures with different computational algorithms within the commonly used software packages R and SAS, and compared the results. The most prevalent fungus was *Leptographium abietinum*, which was recovered from approximately 80% of beetles. The frequency of

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association of both *L. abietinum* and a *Pesotum* species varied with insect population phase in 2001. The presence of *Pesotum* spp. on spruce beetles was negatively associated with the presence of *L. abietinum*. The variance components models indicated that there was significant variation among beetle–fungal associations within trees and trees within sites. Site was generally the smallest source of variation, but estimates were not very precise. We demonstrate how these estimates of variation can be used to design practical sampling protocols to test other hypotheses. This analysis also highlights how attention must be given to understanding experimental design and analysis, and how conflicting conclusions can emerge from hypothesis testing of ecological factors. Our model can be extended as a general analytical approach for future hypothesis testing of bark beetle–fungal associations.

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1. Introduction

Bark beetles (Coleoptera: Curculionidae: Scolytinae; formerly Coleoptera: Scolytidae) that colonize the stems of living conifers undergo intermittent population eruptions that cause region-wide economic losses and exert major landscape-level effects (Mawby et al., 1989; Wermelinger, 2004; Aukema et al., 2005). Most, and perhaps all, bark beetles are intimately associated with a diverse suite of organisms such as mites (Moser and Macías-Sámano, 2000), nematodes (Langor, 1991), protozoa (Wegensteiner et al., 1996), viruses (Wegensteiner and Weiser, 1995), bacteria (Bridges et al., 1984), and fungi (Paine et al., 1997). Relationships between beetles and fungi range from beneficial to antagonistic. For example, fungi benefit from transport, often within specialized cuticular pits on the beetles' heads ("mycangia"), prosterne, or elytra (Lewinsohn et al., 1994; Paine et al., 1997; Six and Paine, 1999). Other fungi may increase brood production by reducing the defensive capacity of host trees (Raffa and Berryman, 1983) or even killing them (Christiansen, 1985; Leufven, 1991; Solheim and Safranyik, 1997). Fungi may also contribute to the production of aggregation pheromones (Brand et al., 1976, 1977), competitively displace antagonistic fungi (Barras, 1970), and/or aid in nutrition (Barras and Hodges, 1969; Barras, 1973; Coppedge et al., 1995; Six, 2003). Alternatively, some fungi may compete with larvae for resources, thereby causing substantial reductions in their development and survival (Barras, 1970; Fox et al., 1992).

Due to interspecific differences in pathogenicity and competitive interactions (Klepzig and Wilkens, 1997), it has been hypothesized that fungi may play an important role in the population dynamics of bark

beetles (Bridges, 1983; Stephen et al., 1983; Harding, 1989; Ohsawa et al., 2000; Wegensteiner and Weiser, 2004). However, the nature of this role is a matter of intense debate (Klepzig and Six, 2004; Eckhardt et al., 2004). For example, it has been proposed that *Ceratocystis polonica* Siem., a phytopathogenic associate of *Ips typographus* L., may be more common during beetle outbreaks, whereas the less virulent *Ophiostoma bicolor* Davidson & Wells may be more prevalent at endemic levels (Solheim, 1993). In contrast, outbreaks of southern pine beetle have been proposed to become more likely when beetles "escape" from the fungus *O. minus* (H. & P. Sydow), which competes with the brood (Bridges, 1983). The degree to which particular fungi and bark beetles are associated is likewise sometimes in dispute, as with *Ceratocystis rufipenni* Wingfield, Harrington, & Solheim, for example (Solheim and Safranyik, 1997; Solheim and Krokene, 1998; Ohsawa et al., 2000; Six and Bentz, 2003). In order to resolve these issues, at least two types of information are needed: (a) an increased knowledge of the multiple mechanisms by which various fungi affect beetle performance, including net effects and context-dependent relationships (Kopper et al., 2004; Eckhardt et al., 2004; Klepzig et al., 2005), and (b) increased quantitative knowledge on how the frequencies of various associates vary with aspects of beetle biology, such as population phase, host species, and geographic range.

This study addresses the latter issue. Quantitatively testing whether frequencies of association of fungi vary with the population phase of their beetle vector, or other factors such as host tree species and geographic range, poses substantial operational and analytical challenges. One must first quantify (a) the

frequency of fungal associations, (b) the amount of variation inherent to the system, and (c) the amount of variation that can be explained by experimental factors (Møller and Jennions, 2002). Failure to account for such variation may obscure potentially important experimental signals. For example, approaches such as computing correlation coefficients between isolation frequency and beetle damage at the site level (Sallé et al., 2005) do not account for other potentially important sources of variation such as the trees from which the beetles originated and provide no flexibility for examining other contributing factors.

Variation in estimated frequencies of specific fungi associated with bark beetles may arise from two sources. The first is from fixed effects, i.e., the experimental factor(s) of interest, such as insect population phase or host tree species. The second comes from random effects, i.e., the experimental units themselves, in this case, the beetles. Random effects may have different levels of nesting. For example, beetles may be sampled from randomly selected trees from randomly selected sites. Experimental models that incorporate both fixed and random effects are termed mixed models, and have certain advantageous properties. Specifically, they allow estimation of the relative sizes of variation of the nested experimental units, or variance components (Searle et al., 1992), which has practical benefits. The relative magnitudes of variation among beetles, trees, and sites, for example, may influence pragmatic and financial decisions when designing sampling protocols. Further, not all beetles within a tree may be entirely independent, e.g., if they originate from the same parents within the same gallery. Lack of complete independence may also be true of trees within a site, or sites themselves. In such cases, mixed effects models are advantageous because they account for random variation within each nested experimental level. Finally, by partitioning the variation among the factors of interest, mixed effects models allow inferences to be drawn about all possible selected experimental subjects. If the beetles, trees, and sites are a truly representative sample of all those possible, the conclusions of hypothesis testing are robust for the entire population.

The probability that a beetle carries a specific fungus can be modeled using logistic regression, in which the binomial response is presence/absence of

the fungus. Logistic regression that incorporates both fixed and random effects is a subclass of generalized linear mixed models (Breslow and Clayton, 1993), in which maximum likelihood estimates for each parameter are estimated using iterative procedures (Schall, 1991). There are several algorithms used to implement such procedures, however, and differences among software programs may yield different answers for approximate tests involving the parameters. This has two notable implications. First, hypothesis tests of the fixed effects, i.e., the factor(s) of interest, may be anti-conservative. Second, parameter estimates may be poor for rare events (Breslow and Clayton, 1993). Thus tests of fungi that may be infrequently associated with bark beetles under some conditions can be unreliable.

The spruce beetle, *Dendroctonus rufipennis* (Kirby), occurs throughout North America where there is contiguous white spruce (*Picea glauca* (Moench) Voss), Sitka spruce (*Picea sitchensis* (Bong.) Carr.), their hybrid Lutz spruce (*Picea lutzii* Little), or Engelmann spruce (*Picea engelmannii* Parry). In the western United States and Canada, it intermittently undergoes immense region-wide outbreaks. For example, during the 1990s, it killed approximately 2.3 million hectares of spruce forests in Alaska and 3 million mature spruce in Utah (Dymerski et al., 2001). Its life cycle is typically 2 years, although it completes development in 1 year under warm conditions. Females bore through the bark of host trees and release aggregation pheromones that attract large numbers of conspecifics. At endemic population levels, beetles colonize primarily windthrown trees, defective trees, and logging slash, but at high population levels they attack relatively vigorous trees. After mating, females oviposit in galleries. First and second instars feed gregariously, and third and fourth instars construct separate feeding galleries. Pupation lasts approximately 2 weeks. Adults move to overwintering sites, under the bark beneath the snow line at the base of infested trees. Insects emerge in the early summer to attack new hosts (Werner et al., 1977; Holsten et al., 1999).

The spruce beetle does not have mycangia. However, spruce beetles are commonly associated with a variety of fungi, predominantly *Leptographium abietinum* (Peck) Wingfield, but also *Ceratocystis coerulea* (Munch) Baski (Reynolds, 1992; Six and Bentz, 2003) (Davidson, 1954, 1955; Hinds and

Buffam, 1971; Ohsawa et al., 2000). Recent work on the *C. coerulescens* complex has identified multiple taxa (Wingfield et al., 1997), including *C. rufipenni* Wingfield, Harrington, and Solheim, which occurs in certain areas (Solheim and Safranyik, 1997; Ohsawa et al., 2000).

The objectives of this study were to determine the frequency of association of various fungi associated with the spruce beetle in Alaska, and to facilitate future studies on the roles of fungi in bark beetle–host associations. We used a multilevel binomial generalized linear mixed model, implemented using different software programs, to estimate the prevalence of each isolated fungal species. We then examined factors that might affect the frequency of association of each fungus with spruce beetles. Specifically, we examined insect population phase, and evidence of possible mutualistic or antagonistic interactions with other fungi. Finally, we examined sources of variation in the association of various fungi with spruce beetles. Inter-beetle, -tree, and -site variation was examined to aid in development of future sampling protocols. We identify cautions in fitting generalized linear mixed models to biological data and compare software packages for their consistency in quantifying abundance and changes in frequency of fungal associates of beetles.

2. Methods

2.1. Collection and isolation protocols

Fungi were isolated from spruce beetles collected from eleven sites in south-central and interior Alaska from 1999 to 2001. Ten sites were located throughout the Kenai Peninsula, and one site, part of an NSF Long Term Ecological Research project, was located near Fairbanks (Aukema, 2003). Each site was separated by more than the estimated 5 km dispersal range of *D. rufipennis* (Beckwith, 1972; Werner and Holsten, 1997). All sites had predominantly (>75%) *P. glauca* or *P. lutzii* (i.e., *P. glauca* × *P. sitchensis* hybrid) overstory. Each collection site was designated as one of four categories: endemic, transition, outbreak, or post-outbreak. These designations were based on data supplied by the USDA Forest Service, and were modified after Wallin and Raffa (2004).

Endemic sites had <10% cumulative tree mortality, and the presence of viable populations was confirmed by examination of colonized hosts. Outbreak sites had >50% of the mature spruce colonized by *D. rufipennis*, and were in areas where the beetles were colonizing vigorous trees at landscape level scales. Transition sites had between 10 and 50% mortality, and post-outbreak sites had near 100% mortality.

We performed a variety of collection and microbial isolation techniques during the methods refinement phase of this study, to assure qualitative depiction of the fungi present, and devise appropriate methods for quantitative analyses. Insects were collected via two methods: mass-trapping in multiple funnel traps baited with the aggregation pheromone frontalin (Gries et al., 1988) and the host monoterpene α -pinene (Furniss et al., 1976), and excavations of overwintering adults from around the bases of host trees prior to flight. Following excavations, insects from a specific tree were placed in the same vial in 1999 and 2000, but more frequently in separate vials in 2001. This allowed comparison of effects of pre-plating storage density on isolation frequencies. Collected insects were stored at 4 °C and shipped overnight to the USDA Forest Products Laboratory in Madison, Wisconsin, where fungi were isolated.

Isolations were performed by rolling and by dilution plating. In the former method, insects were rolled across the medium with sterile forceps. In the dilution plating method, one insect was ground with a sterile glass tissue homogenizer in 9 ml of sterile water (Juzwik and French, 1983; Klepzig et al., 1991; Haberkern et al., 2002). Five hundred microliters of successive 10^{-2} , 10^{-3} , and 10^{-4} dilutions of the liquid homogenate in sterile water were evenly spread on individual plates with a sterile glass rod (Haberkern et al., 2002). Fungi were cultured in 100 mm × 15 mm Petri dishes on a variety of media including malt extract agar, potato dextrose agar, and Taylor's medium, amended with the antibiotics tetracycline and gentamicin, in separate trials. Incubation temperatures ranged from 10 to 27 °C. In addition to the beetles, we sampled host phloem tissue and host sapwood tissue for fungi.

We chose to primarily excavate adult beetles from overwintering sites, and perform isolations by rolling

the beetles across 1.5% malt extract agar amended with 1 µg/ml gentamicin, in 2000 and 2001. Our rationale for excavating individual insects was (a) greater recovery of the primary associate *L. abietinum* (Fisher's exact test, see Section 3), (b) removal of biases introduced by insect death in traps (see Section 3), and (c) ability to identify the exact tree in which beetles developed. Our rationale for rolling beetles, rather than dilution plating was (a) equivalent frequencies of fungal isolation for the most prevalent species (Fisher's exact test, see Section 3) (b) identical fungal species richness or diversity (see Section 3), and (c) the rolling procedure was substantially less labour intensive than serial dilution plating, and hence more amenable to obtaining adequate sample sizes for statistical analysis. Further details on methodological permutations and their results can be found in Aukema (2003).

Hyphal morphology and characteristics of the conidiophores and conidia were used to distinguish ascomycetous fungi and classify them into putative types (de Hoog, 1993; Kendrick et al., 1993; Wingfield, 1993; Upadhyay, 1993; Siefert and Okada, 1993). These types were transferred to new Petri dishes, incubated for 1 week at 22 °C, and then stored at 4 °C until identified (Haberkern et al., 2002). Identifications were based on colony, anamorph, and teleomorph morphology (Olchowecki and Reid, 1974; Nag Raj and Kendrick, 1975; Upadhyay, 1981; Grylls and Siefert, 1993). Fruiting structures were examined by mounting on glass slides and examining with light microscopy similar to Haberkern et al. (2002). Identifications were confirmed by Thomas Harrington (Department of Plant Pathology, Iowa State University).

2.2. Statistical model framework

The binary response, i.e., presence/absence of each fungus, was examined using a generalized linear mixed effects model for each year. This model uses the logit transformation characteristic of logistic regression (a natural logarithmic transformation of the odds ratio (McCullagh and Nelder, 1989)) to express the probability of a specific fungus occurring on a randomly selected beetle. Covariates may include fixed effects, such as beetle population phase and/or the presence of other specific fungal species, and

random effects of experimental sites, trees, and vials. Eq. (1) lists a general framework:

$$\text{logit}(p_{prs,jkl}) = \ln\left(\frac{p_{prs,jkl}}{1 - p_{prs,jkl}}\right) = \mu + \text{phase}_p + \text{sp}_r + \text{sp}_s + \dots + \text{site}_l + \text{tree}_{kl} + \text{vial}_{jkl} \quad (1)$$

where μ estimates an overall mean frequency of fungi on all possible beetles, p indexes differences in insect population phase categories (endemic, transition, outbreak, and post-outbreak), sp_1 , sp_2 , etc. are the presence/absence of other specific co-occurring fungi, and j , k , and l index the nested random effects, i.e., collection vials, trees, and sites, respectively. This framework can be extended to examine interactions between insect population phase and co-occurring fungi, or test other covariates of interest such as spatial location. When examining fixed effects, we used a backward elimination procedure, setting $\alpha = 0.05$, in tandem with examination of Akaike's information criteria while maintaining similar random effects (Akaike, 1973; Pinheiro and Bates, 2002). When examining potential competitive effects due to specific fungi, we only included fungi present on >3% of the insects. An effect for the variation due to the number of insects placed in vials was included in 2001 analyses only. The relative variation of each random effect was studied by examining a measure of spread, such as its standard deviation or 95% confidence interval, on the scale of the linear predictor (right-hand side of Eq. (1)). The significance of inter-site variation, which was frequently quite low (see Section 3), was assessed using likelihood ratio tests between nested models with and without site. Because tests of random effects were based on comparisons of restricted maximum likelihood, each test maintained the same chosen fixed effects structures (Pinheiro and Bates, 2002).

2.3. Comparisons among software packages

Because generalized linear mixed effects models are relatively new (<10 years), developing efficient computational implementations that yield consistent, reliable estimates is a subject of ongoing research (Wolfinger, 1993; Wolfinger and O'Connell, 1993; Vonesh, 1996; McCulloch, 1997; Raudenbush et al., 2000). Software programs often use different fitting algorithms, and may yield different estimates of beetle–

fungus associations. To investigate potential discrepancies, two different software programs, R v1.7.1 (Ihaka and Gentleman, 1996; R Development Core Team, 2004), and SAS v8.2 (SAS Institute, Cary, NC), were used to estimate the frequency of spruce beetle–fungi associations. These programs were selected because of their popularity and because they are among the few that contain procedures to implement multiple levels of nesting in generalized linear mixed models. In R, two different procedures were used: glmmPQL (MASS package) and GLMM (lme4 package). In SAS, the glimmix macro was used. This macro uses processing statements similar to Proc Mixed (Littrell et al., 1996). GLMM, glmmPQL, and the glimmix macro each use various penalized quasi-likelihood approximations to calculate maximum likelihood estimates (Wolfinger and O’Connell, 1993; Breslow and Clayton, 1993). To compare each procedure, we examined parameter estimates, confidence intervals (if applicable), asymptotic convergence of the estimates, and tests of the effects in the model (Eq. (1)).

3. Results

3.1. What fungi are associated with *D. rufipennis* in Alaska?

We isolated 10 different fungi from approximately 1000 adult spruce beetles over 3 years. Because we used 1999 for protocol development (Aukema, 2003), we focus on 2000 and 2001 data in this paper (Table 1). *L. abietinum* was the most prevalent, and was found on more than 70% of the insects in 2000 and 2001. We found *Ophiostoma* sp. A on 5 of 331 (12.7%) beetles in 2000, but on only one of 466 insects the following year. *Pesotum* sp. C was found on approximately 5% of the beetles each year. *Pesotum* sp. F was found on 6% of insects in 2000, and increased to 19% in 2001. *Ophiostoma picea* was found on 3.0% of the beetles in 2000. All other fungi were found on 3 or fewer insects in a given year.

Isolations of *L. abietinum* were more successful with individually excavated beetles than beetles caught in traps. In 2000, 86% of 246 live excavated insects carried this fungus versus only 51% of 45 live trapped insects (Fisher’s exact test, $P < 0.0001$). Method of insect acquisition had no significant effect

Table 1

Fungi isolated from live individual spruce beetles collected in Alaska, 2000 and 2001

Fungus	2000		2001	
	<i>n</i> = 331	%	<i>n</i> = 466	%
<i>L. abietinum</i>	269	81.27	332	71.24
<i>O. cainii</i>	3	0.91	1	0.21
<i>O. picea</i>	10	3.02	0	0.00
<i>Ophiostoma piceaperdum</i>	0	0.00	1	0.21
<i>Ophiostoma</i> sp. A	42	12.69	1	0.21
<i>Ophiostoma</i> sp. D	3	0.91	1	0.21
<i>Ophiostoma</i> sp. E	0	0.00	0	0.00
<i>Pesotum</i> sp. C	16	4.83	27	5.79
<i>Pesotum</i> sp. F	20	6.04	88	18.88
Unknown A	0	0.00	1	0.21
No fungus isolated	44	13.29	48	10.30

on the detection of any other fungi ($P > 0.05$). We evaluated dead trapped insects separately, and observed a strong bias due to beetle mortality. For example, in 2001, we were unable to culture *L. abietinum* from 18 beetles captured in flight traps, all of which were dead when plated. There was no significant difference in isolation frequency of the most prevalent fungi between the two isolation methods, rolling beetles and dilution plating (Fisher’s exact test for 1999 data: *L. abietinum*, $P = 0.259$; *Pesotum* sp. F, $P \approx 1$). There was no difference in species richness or species composition between the two methods. Neither method yielded any fungi that were not yielded by the other.

We applied the logistic model (1) to the 2000 and 2001 data to estimate the mean prevalence of each fungus each year. In estimating the mean frequency, i.e., μ , we excluded fixed effects (e.g., adjustments for potential explanatory factors such as insect population phase), because we sampled from sites representative of all phases and the model weights sample sizes accordingly. Estimated mean frequencies of association of each fungus with spruce beetles are listed in Table 2. All estimates were highly significant ($P \leq 0.0004$). The predominant associate, *L. abietinum*, was estimated to occur on 79.5 and 72.7% of the insects in 2000 and 2001, respectively. These estimates were relatively consistent with the observed frequencies of 81.3 and 71.2%. In contrast, population estimates of frequencies of less abundant fungi were often lower than observed frequencies from our field-collected data. For example, *Ophiostoma* sp. A was

Table 2

Estimation of overall frequency of association (i.e., μ) of various fungi with spruce beetles in Alaska, 2000 and 2001

Year	Fungus	Fitted estimate (%) ^a			Observed frequency(%)	d.f. ^b			Significant random effects ^c		
		glmmPQL	glimmix	GLMM		glmmPQL	glimmix	GLMM	glmmPQL	glimmix	GLMM
2000	<i>L. abietinum</i>	79.50	79.49	79.50	81.27	303	27	302	Tree	Tree	Tree
	<i>O. picea</i>	NA ^d	0.56	0.76	3.02	303	27	302	Tree	Site, tree	Site, tree
	<i>Ophiostoma</i> sp. A	7.19	7.02	7.19	12.69	303	27	302	Tree	Tree	Tree
	<i>Pesotum</i> sp. C	2.50	2.40	2.50	4.83	303	27	302	Tree	Tree	Tree
	<i>Pesotum</i> sp. F	3.55	3.45	3.55	6.04	303	27	302	Tree	Tree	Tree
2001	<i>L. abietinum</i>	72.73	72.79	72.73	71.24	229	30	228	Tree, vial	Tree, vial	Tree, vial
	<i>Pesotum</i> sp. C	2.15	1.99	3.72	5.79	229	7	228	Site, tree, vial	Site, tree, vial	Site, tree, vial
	<i>Pesotum</i> sp. F	11.70	11.26	11.41	18.88	229	7	228	Site, tree, vial	Site, tree, vial	Site, tree, vial

^a Estimate on the scale of the response, $\left(\frac{1}{1+e^{-\mu}}\right)$. This is a back transformation of Eq. (1) (without fixed effects).^b Denominator degrees of freedom (numerator degrees of freedom for estimating intercept μ is 1).^c Likelihood ratio tests test whether site is a significant source of variation (see Table 4).^d Estimate failed to converge.

isolated from 12.7% of the insects in 2000, while the highest model estimate was 7.2%. *Pesotum* sp. F was isolated from 18.9% of the insects in 2001, but the highest model estimate was 11.7%. Estimates of fungi isolated $\leq 5\%$ of the time varied greatly between software packages, or were unattainable. For example, *Pesotum* sp. C was isolated from 5.8% of insects in 2001, but it was estimated to occur on between 2.0 and 3.7% of the insects. Similarly, *O. picea* was isolated from 3.0% of the insects in 2000, but model estimates from both glimmix and GLMM procedures were less than 1% for that year. Further notes on software package comparisons are provided in Section 3.4.

3.2. Does the frequency of specific fungal associations with spruce beetle vary with population phase and/or the presence of other fungi?

The frequency of association of *L. abietinum* and *Pesotum* sp. F with the spruce beetle varied significantly among different beetle population phases in 2001 (*L. abietinum*: $F_{3,27} = 3.45$, $P = 0.0304$ using glmmPQL; $F_{3,229} = 2.67$, $P = 0.0481$ using the glimmix macro; $F_{3,462} = 2.16$, $P = 0.0919$ using GLMM; *Pesotum* sp. F: $F_{3,27} = 3.90$, $P = 0.0196$ using glmmPQL and $F_{3,229} = 3.28$, $P = 0.0218$ using the glimmix macro; $F_{3,462} = 2.62$, $P = 0.0505$ using GLMM). The relationship between insect population phase and the frequency of association with these

fungi is shown in Fig. 1. *L. abietinum* was present on approximately 82% of the beetles in endemic or outbreak sites, but on only 62% of the beetles on sites undergoing pre- or post-outbreak transition. In contrast, *Pesotum* sp. F was present on approximately 10% of spruce beetles in endemic or outbreak sites. The frequency of association was 28% in sites undergoing transition to outbreak phase, and those following outbreak population phases. *L. abietinum* was approximately eight times more abundant than *Pesotum* sp. F in endemic and outbreak locations. We

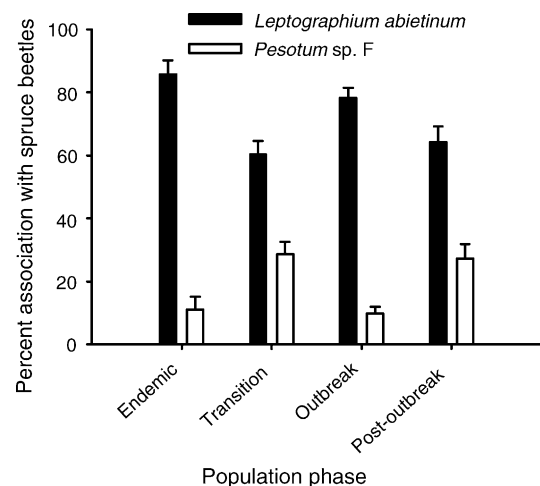


Fig. 1. Relationship of spruce beetle population phase to the observed (+S.E.) frequencies of association of *L. abietinum* and *Pesotum* sp. F with spruce beetles in Alaska, 2001.

did not find similar patterns in 2000, or for other fungal species (P -values ranged from 0.10 to 0.87).

The inverse association between *L. abietinum* and *Pesotum* sp. F across spruce beetle population phases (Fig. 1) also occurs at the level of individual beetles (Table 3). The probability that an individual beetle carried *L. abietinum* decreased with the co-occurrence of *Pesotum* sp. F, as well as *Ophiostoma* sp. A and *Pesotum* sp. C. For example, in 2001, if a beetle carried *Pesotum* sp. F, there was only a 20% chance that it also carried *L. abietinum*, even though approximately 85% of beetles without *Pesotum* spp. or *Ophiostoma* sp. A carried *L. abietinum* (Table 3). Other fungal species examined did not affect the frequency of association of *L. abietinum* at $\alpha = 0.05$. In general, models containing co-occurring fungi were not improved by the addition of a term for insect population phase, and vice versa. For example, *L. abietinum* and *Pesotum* sp. F are negatively correlated, regardless of population phase.

3.3. What are the relative sources of variation among experimental units?

Variance components were estimated for all nested random effects (i.e., site, tree, and insect collection vial) in models containing only a mean frequency μ

(as in Table 2). Estimates of variation among the random effects are listed in Table 4. Variation among sites was typically very low for relatively abundant fungi such as *L. abietinum* and *Pesotum* spp. in 2000, and *L. abietinum* in 2001 (Table 4: likelihood ratio tests). In 2001, the inter-site variation in *L. abietinum* association with spruce beetles was approximately half of the inter-tree (within site) variation, which in turn was approximately half of the vial-to-vial (within tree) variation. Site was found to be a significant source of environmental variation in 2001 for the *Pesotum* spp., being slightly more than or approximately equal to inter-tree variation within each site. Within-tree differences among beetles contributed the largest source of variation for all fungi among the random effects in 2001.

3.4. Comparisons among software packages

There were two notable differences between software packages. First, the degrees of freedom in the denominator differed when conducting approximate tests for the significance of estimated model parameters. Such parameters included the mean frequency of a specific fungus in the beetle population, or coefficients for fixed effects such as insect population phase. For example, when estimating an overall mean,

Table 3

Probability that a spruce beetle carries *L. abietinum*, given the co-occurrence of *Pesotum* spp. C and F and *Ophiostoma* sp. A

Year	Co-occurring fungi ^a			Model estimate (%) ^b			Observed ^c		
	<i>Pesotum</i> sp. C	<i>Pesotum</i> sp. F	<i>Ophiostoma</i> sp. A	glmmPQL	glimmix	GLMM	Positive	<i>n</i>	Frequency (%)
2000				84.33	84.34	84.33	251	298	84.23
	x			56.15	55.81	56.15	10	16	62.50
	x			42.07	41.89	42.07	9	20	45.00
	x			68.48	68.50	68.48	27	42	64.29
	x	x		14.73	14.46	14.73	1	3	33.33
	x		x	34.06	33.77	34.06	2	2	100.00
		x	x	22.67	22.54	22.67	3	6	50.00
	x	x	x	6.52	6.39	6.52	0	0	0.00
2001				84.72	84.77	84.72	303	353	85.84
	x			38.91	38.71	38.90	12	27	44.44
		x		21.70	21.85	21.70	17	88	19.32
	x	x		3.09	3.08	3.09	0	0	0.00

^a Co-occurrence of various fungi indicated with an 'x' across rows. *Ophiostoma* sp. A was not examined in 2001, as it was only found on one insect (Table 1). Rows without co-occurring fungi indicate the prevalence of *L. abietinum* on beetles without *Pesotum* spp. or *Ophiostoma* sp. A.

^b Estimate on the scale of the response, $\left(\frac{1}{1 + e^{(-\mu + \text{Psp } C_r + \text{Psp } F_s + \text{Osp } A_t)}} \right)$. This is a back transformation of Eq. (1).

^c Observed frequencies, calculated as the number of beetles that had both *L. abietinum* and the co-occurring fungi, i.e. the positive column, divided by *n*, the number of beetles associated with the co-occurring fungi listed.

Table 4

Summary of variance estimates of site, tree, vial, and residual scaling parameter when examining the overall frequency of fungal associations with spruce beetles in Alaska, 2000 and 2001

Year	Fungus	Nested random effect ^a	Estimate of standard deviation, $\hat{\sigma}$			95% confidence intervals about $\hat{\sigma}^b$		Likelihood ratio tests ^c					
			glmmPQL	glimmix	GLMM	Lower	Upper	χ^2_I			P		
								glmmPQL	glimmix	GLMM	glmmPQL	glimmix	GLMM
2000	<i>L. abietinum</i>	Site	0.03	0.00	0.02	5.35×10^{-11}	1.77×10^7	0.07	0.00	0.00	0.7845	1.0000	0.9724
		Tree	1.14	1.19	1.26	0.71	1.84						
		Residual	0.91	0.91	0.95	0.84	0.98						
	<i>O. picea</i>	Site	1.30×10^6	1.72	2.85	NA ^d	NA	0.00	101.30	272.51	1.0000	<0.0001	<0.0001
		Tree	4.11×10^6	1.76	0.00	NA	NA						
		Residual	1.15×10^7	0.51	0.81	NA	NA						
	<i>Ophiostoma</i> sp. A	Site	0.01	0.00	0.02	2.20×10^{-23}	8.35×10^{18}	0.13	0.00	0.00	0.7169	0.9998	0.9840
		Tree	1.42	1.47	1.79	0.93	2.15						
		Residual	0.79	0.79	0.89	0.73	0.86						
	<i>Pesotum</i> sp. C	Site	0.06	0.00	0.03	1.23×10^{-7}	2.52×10^4	0.13	0.00	0.00	0.7142	0.9998	0.9549
		Tree	1.47	1.56	2.09	0.89	2.42						
		Residual	0.70	0.70	0.84	0.65	0.76						
	<i>Pesotum</i> sp. F	Site	0.06	0.00	0.02	2.74×10^{-7}	1.22×10^4	1.08	0.00	0.00	0.2995	0.9999	0.9774
		Tree	1.09	1.13	1.38	0.67	1.78						
		Residual	0.78	0.78	0.89	0.72	0.85						
2001	<i>L. abietinum</i>	Site	0.11	0.27	0.18	7.08×10^{-13}	1.76×10^{10}	0.32	0.30	0.13	0.5698	0.5813	0.7209
		Tree	0.58	0.55	0.66	0.13	2.61						
		Vial	1.35	1.37	1.60	1.02	1.78						
		Residual	0.84	0.84	0.92	0.78	0.92						
	<i>Pesotum</i> sp. C	Site	1.10	1.30	1.05	0.42	2.92	47.20	60.04	186.00	<0.00001	<0.0001	<0.0001
		Tree	0.66	0.65	1.00	0.16	2.77						
		Vial	2.13	2.15	0.00	1.73	2.61						
		Residual	0.59	0.59	0.89	0.55	0.63						
	<i>Pesotum</i> sp. F	Site	0.53	0.60	0.71	0.17	1.60	6.42	7.27	5.00	0.0113	0.0070	0.0253
		Tree	0.62	0.63	0.84	0.23	1.71						
		Vial	1.63	1.65	2.19	1.31	2.03						
		Residual	0.74	0.74	0.86	0.69	0.80						

^a Residual term in nested random effects is a variance scaling parameter ϕ (see Section 4).

^b 95% confidence intervals are provided by glmmPQL. Intervals are constructed on a logarithmic scale to induce normality, and then back-transformed.

^c Likelihood ratio tests test whether site, frequently the lowest variance term, is a significant source of variation.

^d Estimate failed to converge.

μ , glmmPQL and GLMM frequently used many more degrees of freedom (up to 300) than the SAS glimmix macro (up to 30) (middle columns of Table 2). When testing the effect of insect population phase on the frequency of fungal association in 2001, glmmPQL used the lowest denominator degrees of freedom (27), even when all three procedures had identical random effects structures. The other procedures used up to 229 (glimmix) and 462 (GLMM) degrees of freedom.

The second notable difference was in convergence among the different iterative algorithms implemented by the software. For example, in 2000, *O. picea* was estimated to occur on 0.56% of adult spruce beetles in Alaska, according to the model estimates of the SAS glimmix macro (Table 2). The macro ran correctly, although convergence criteria were not met, as noted in the log file. The list file generated by SAS procedures incorrectly stated that convergence criteria were met. Estimates using glmmPQL could not be attained due to nonconvergence. Even when estimates were obtained, the precision could be poor. For example, when estimating variance among sites (Table 4), confidence intervals reported by glmmPQL could vary over 10 orders of magnitude.

4. Discussion

4.1. Fungi associated with spruce beetles in Alaska

The complex of fungi associated with spruce beetle was dominated by *L. abietinum* (Tables 1 and 5),

consistent with other studies (Davidson, 1955; Hinds and Buffam, 1971; Ohsawa et al., 2000; Six and Bentz, 2003). This domination of the flora of its vector differs from *L. abietinum*'s association with the Douglas-fir beetle *D. pseudotsugae* Hopkins, from which *O. pseudotsugae* (Rumbold) von Arx is also a frequent associate (Harrington, 1988; Lewinsohn et al., 1994; Ross and Solheim, 1997; Solheim and Krokene, 1998). *Pesotum* spp., *Ophiostoma cainii*, and possibly *Ophiostoma* spp. A, D, and E represent newly reported associations with the spruce beetle.

We did not observe *O. tricolor* Davidson, *O. bicolor* Davidson & Wells (Davidson, 1955), *O. olivaceum* Mathiesen (Hinds and Buffam, 1971), *O. piliferum* (Fries) H. & P. Sydow (Safranyik et al., 1983), *O. ips* (Rumbold) Nannf. (Six and Bentz, 2003), or *C. rufipenni* (Davidson, 1955; Hinds and Buffam, 1971; Safranyik et al., 1983), which have been observed in some *D. rufipennis* populations. *C. rufipenni* is highly virulent (Solheim and Safranyik, 1997), but appears primarily localized in British Columbia (Safranyik et al., 1983), sporadically in Alberta (Ohsawa et al., 2000) and possibly in Colorado (Davidson, 1955) (if the *C. coerulescens* forms isolated by Davidson included *C. rufipenni*; Wingfield et al., 1997). Our results concur with previous studies that did not locate *C. rufipenni* in the Kenai Peninsula of Alaska (Reynolds, 1992; Six and Bentz, 2003), Colorado and Utah (Six and Bentz, 2003), Minnesota (Haberkern et al., 2002; Six and Bentz, 2003), Wisconsin and Michigan (Haberkern et al., 2002). We followed procedures that have isolated this fungus where present (Solheim and Krokene, 1998), and we supplemented our methods with a broad range

Table 5

Estimates of variation per spruce beetle sampled about the estimated mean frequency of association of *L. abietinum*

Number of sites	Beetles per vial	Pooled variance estimate ^a				Overall cost of sampling scheme (US\$) ^b			
		6 trees		12 trees		6 trees		12 trees	
		1 vial	12 vials	1 vial	12 vials	1 vial	12 vials	1 vial	12 vials
6	1	0.1759	0.0251	0.0889	0.0135	1872.60	2011.20	2245.20	2522.40
6	12	0.0715	0.0164	0.0367	0.0092	1912.20	2486.40	2324.40	3472.80
12	1	0.0879	0.0125	0.0445	0.0068	3745.20	4022.40	4490.40	5044.80
12	12	0.0357	0.0082	0.0184	0.0046	3824.40	4972.80	4648.80	6945.60

These estimates are shown for different hypothetical numbers of beetles, vials, trees, and sites sampled. To illustrate the utility of this approach, the overall cost of sampling schemes associated with different allocations of sampling effort is shown.

^a Based on 2001 data ($n = 466$ insects). Beetle variation $\hat{\sigma}_e^2 = 4.10$ (Eq. (3); Table 4), vial variation $\hat{\sigma}_j^2 = 1.82$ (Table 4), tree variation $\hat{\sigma}_k^2 = 0.34$ (Table 4), and site variation $\hat{\sigma}_l^2 = 0.012$ (Table 4).

^b Cost for sampling each unit: cost per beetle, $c_i = \text{US\$ } 0.10$; per vial, $c_j = \text{US\$ } 0.25$; per tree, $c_k = \text{US\$ } 10$; and per site, $c_l = \text{US\$ } 250$ for illustrative purposes, intended primarily for relative comparisons among sampling schemes. Overall cost estimates are derived from Eq. (5).

of culturing temperatures, media, and beetle handling measures (Aukema, 2003). Confirmed *C. rufipenni* has been isolated from galleries and sapwood, but it has never been isolated directly from the spruce beetle (Wingfield et al., 1997; Ohsawa et al., 2000; Six and Bentz, 2003). However, we also sampled galleries and sapwood, and did not observe *C. rufipenni*. It seems unlikely that *C. rufipenni* was present but obscured by other fungi, because evaluation of approximately 1000 insects from multiple sites over 3 years assured that there would likely be some samples without potentially obscuring fungi, given the high beetle to beetle variation.

Some of the less consistently recorded fungi could reflect subsequent colonization of killed trees by secondary beetle species (Six and Bentz, 2003). However, the fungal species compositions of excavated and flying beetles were equivalent.

4.2. Relationship of beetle–fungal associations to insect population phase

Our results provide some support for the view that the frequency of fungal association changes with bark beetle population phase. However, the evidence was inconsistent between years. Several beetle traits have been observed to vary with population density, such as behavior (Wallin and Raffa, 2004) and yolk protein deposition (Thomson and Sahota, 1981; Sahota et al., 1984). Fungal relationships could represent another density-dependent character. In 2000, we did not find evidence of population phase variation in the frequency of fungal associates, but we did find patterns consistent with competition between *L. abietinum* and *Pesotum* sp. F on individual beetles (Table 3). Interestingly, these two fungi demonstrated opposing patterns across insect population phases the following year, which again would be consistent with competition (Fig. 1). Changes in bark beetle–fungal frequencies across insect population phases may involve both competitive shifts among multiple fungi and changes in the relative frequencies of individual fungi.

4.3. Variance components, and implications for sampling methodology and hypothesis testing

The variance estimates from beetle–fungal associations (Table 4) can be used to develop sampling

guidelines for testing other hypotheses, such as the effects of elevation, spatial location, host tree species, or weather on fungal association. For example, given a sufficiently large sample size of beetles, the probability distribution of the number of beetles that carry a specific fungus becomes approximately normal, according to the properties of the Central Limit Theorem. Hence Eq. (1) can be approximately expressed as

$$\text{logit}(p_{ijkl}) = \ln\left(\frac{p_{ijkl}}{1 - p_{ijkl}}\right) \cong \mu + \text{site}_i + \text{tree}_{kl} + \text{vial}_{jkl} + \varepsilon_{ijkl} \quad (2)$$

where $\varepsilon_{ijkl} \sim N(0, \sigma_\varepsilon^2)$ and ε_{ijkl} describes the beetle to beetle variation. Note that the fixed effects have been removed, so Eq. (2) examines the relative variability of a specific fungus on a specific beetle by partitioning the variance inherent within the system solely among the experimental units. The estimated variance about the estimate of the overall frequency of association of a specific fungus with spruce beetles (i.e., μ) can be expressed as

$$s_{\hat{\mu}}^2 = \frac{\sigma_\varepsilon^2 + i\sigma_l^2 + j\sigma_k^2 + jk\sigma_j^2}{ijkl} \\ = \frac{\sigma_\varepsilon^2}{ijkl} + \frac{\sigma_j^2}{jkl} + \frac{\sigma_k^2}{kl} + \frac{\sigma_l^2}{l} \quad (3)$$

Eq. (3) partitions the relative sources of variation among the sites (σ_l^2), trees (σ_k^2), sampling vials (σ_j^2), and individual beetles (σ_ε^2). Site, tree, and vial variation can be estimated directly (Table 4). Beetle variation can be estimated as

$$\hat{\sigma}_\varepsilon^2 = \phi \left(\frac{1}{\hat{p}(1 - \hat{p})} \right) \left(\frac{1}{n} \right) \quad (4)$$

where ϕ is a residual scaling parameter (Table 4) used when beetle-to-beetle variance differs from the hypothetical asymptotic variance (i.e., over- or under-dispersion), \hat{p} is the estimated frequency of association, and n is the number of subjects per trial (in this case, one beetle per plate) (McCullagh and Nelder, 1989). By partitioning the relative sources of variation, one can determine where to focus sampling efforts, as it is desirable to obtain the greatest amount of sampling effort where variation is greatest (Snedecor and Cochran, 1989).

These outputs of variance comparisons can be used to improve operational efficiency and experimental validity. The left portion of Table 5 lists estimates of variation about the sample mean for *L. abietinum* in 2001 at different hypothetical numbers of beetles, vials, trees, and sites sampled (i.e., inserting variance estimates from Table 4 into Eq. (3)). Not surprisingly, the variation per sample decreases as a greater number of samples are obtained (within columns and across rows). However, in any experiment, one must weigh potential trade-offs in minimizing sampling variance against the potential costs in time and money of collecting additional samples and subsamples (Werner et al., 2004). On the right side of Table 5, we show a simulated cost scheme for the sampling permutations listed. The overall cost of a sampling scheme, C , can be calculated as

$$C = c_i i j k l + c_j j k l + c_k k l + c_l l \quad (5)$$

where c_i , c_j , c_k , and c_l are the costs specific to each insect, vial, tree, and site respectively, and i , j , k , and l index the number of insects, vials, trees, and sites as in previous equations. We think it is reasonable to assume, for example, that it is more costly to establish more research sites than sample more trees within a site. In our simulated cost scheme, we set costs of selecting each beetle, vial, tree, and site as US\$ 0.10, 0.25, 10, and 250 respectively. One might reasonably wish to sample 864 insects from 72 trees, which could be accomplished by sampling 6 trees from 12 sites, or 12 trees from 6 sites. Sampling a dozen insects from each tree and placing them in individual vials, we find that the pooled sample variances per insect are similar (Table 5; 0.0125 versus 0.0135). However, the strategy with 12 sites would cost US\$ 1500 more. Another operational consideration is whether to place insects in separate vials, or pool insects from each tree into a single vial. Intuitively, it seems preferable to store individual insects separately in the field, despite the added effort. A quantitative analysis supports this method: the variation arising from placing 12 beetles in individual vials is always less than half that of placing twelve beetles in one vial, and the costs are always within US\$ 500 (Table 5). The reader should be cautioned that this simulation reflects properties of this particular system. Different systems with different variance components might result in substantially different appropriate sampling schemes. Although our sampling simulation is

performed with *L. abietinum*, the feasibility of any sampling strategy can be calculated for any fungal species associated with *D. rufipennis* (Aukema, 2003).

Examination of variance components lends insight into another operational decision, whether to obtain beetles by excavation or trapping. The latter is logistically easier, but also promotes cross-contamination among insects (Viiri, 1997). Theoretically, beetles congregated within hosts prior to excavation might also contaminate each other. However, beetle-to-beetle variation within trees was at least twice that of all other sources of variation (Table 4), indicating that it is not problematic in this system. Another consideration is the loss of information at the tree level, because trapping obscures the beetle's point of origin. In this system, individual trees are an important source of variation, and so losing this information is disadvantageous. Given the operational ease of pheromone traps, however, and the need to validate that the fungal associates of excavated beetles reflect those of flying beetles, we recommend that studies whose objectives are to both characterize beetle–fungal associations and quantify them for purposes of hypothesis testing employ both excavating and funnel-trapping methods as complementary approaches.

The pooled variance estimates can also be used in power calculations to determine how many sites to sample to realize a true difference δ regarding a hypothesis of interest. For example, suppose one wishes to test whether the frequency of association of *L. abietinum* varies between two host trees such as *P. glauca* versus *P. engelmannii*. Based on preliminary sampling, one might estimate that *L. abietinum* is found on 50% more of the insects colonizing *P. glauca* than *P. engelmannii*. Using Eq. (3) and the variance estimates from Table 4, we calculate the pooled sample variance for a single site (i.e., $j = 1$) for a sampling scheme of k trees, l vials, and i insects. For *L. abietinum* in 2001, sampling 12 insects from each of 12 trees and storing them separately results in a pooled sampling variance of 0.0813. An 80% power calculation using $\alpha = 0.05$ can be approximated as

$$n = \left\lceil \frac{2 \times 7.9 \times s_p^2}{\delta^2} \right\rceil + 1 \quad (6)$$

where n is the number of sites to sample for each treatment (two tree species) to obtain an 80% prob-

ability of detecting a true difference δ . In this equation, s_p^2 is the sampling variance and the constants are derived from independence and normal distribution considerations (Snedecor and Cochran, 1989). From the above example, using $\delta = 0.5$, we find that $n = 6.1$, so seven sites should be selected in each group. This power calculation is a flexible approach that can be easily extended to other insect and fungal species.

4.4. Cautions in model implementation

Care must be used when assessing hypothesis tests in multilevel models due to potential differences in the degrees of freedom calculated by the software. Ignoring the hierarchical structure of data can lead to false inferences, as seen in nested models in the health care literature (Austin et al., 2001). Conservative tests use the degrees of freedom associated with the highest level of nesting, such as site or tree, rather than beetles, as the beetles were subsampled from trees. Surprisingly, the procedure with the highest denominator degrees of freedom (GLMM with 462) provided the only non-significant test when testing the effect of insect population phase on frequency of *L. abietinum* in 2001. This may be an example of a Type II error. In general, we advocate hypothesis testing with conservative (i.e., lowest) degrees of freedom.

Population estimates of the frequency of association of fungi sometimes differed from observed frequencies (Table 2). This was due in part to the logit response transformations (Eq. (1)), but also may have been due to the fitting algorithms. Estimates of the association of *O. picea* with spruce beetles, for example, were difficult to obtain in 2000, when they were found on 3% of the insects. Safranyik et al. (1983) identified *O. picea* as an important associate of spruce beetles, which suggests that fungi that are relatively uncommon in certain conditions may be important in hypothesis testing. Estimates for *O. picea* were still difficult to obtain if we increased the default number of fitting iterations in glmmPQL or provided different starting variance estimates in the SAS glimmix macro. Computational fitting algorithms that may provide better estimates exist (Wolfinger, 1993; Vonesh, 1996; Raudenbush et al., 2000; Evans and Swartz, 2000), but software packages that use such algorithms may limit nesting structures, such as SAS Proc Nlmixed. Future research will benefit from

software currently in development, and other techniques such as Bayesian hierarchical modeling, which allows multiple levels of nesting and greater choice of fitting algorithms.

4.5. Implications to forest management, and to vector-microbe systems

While we found some evidence of an association of insect population phase with the frequency of association of *L. abietinum* and a *Pesotum* species, and relationships consistent with competitive interactions between these two species across population phases, long-term, site-specific studies over the transition of multiple insect population phases are needed to elucidate the mechanisms responsible for the observed patterns. Other issues requiring investigation include the effects of competitors of scolytids and stand heterogeneity on beetle–fungal interactions. It is possible that management strategies for spruce beetle that incorporate stand heterogeneity may affect secondary scolytids, and potentially primary beetles and their fungal complement as well.

Our approach of examining variation at multiple levels of scale to inform sampling efforts and test specific hypotheses may be extended to other bark beetle and insect vector systems. For example, this approach may be used to detect clusters of animal or plant disease at different nested spatial scales, or test whether hosts affect the frequency of disease incidents.

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